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TITLE: Super p53 for Treatment of Ovarian Cancer

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

In this report, we show preliminary data indicating that gene therapy using re-engineered super p53 (p53-CC constructs) is effective in killing ovarian cancer cells in vitro. This is unreported, novel finding paves the way for using super p53 for ovarian cancer treatment. Main activities and objectives completed to date include optimization of cell culture growth and determining transfection conditions for human ovarian cancer cell lines, including Kuramochi, OVCAR3, SKOV3, SKOV3.ip1, and mouse ovarian cancer cells (ID8). 7AAD and MTT assay conditions have been optimized. WSLP (polymer) has been successfully synthesized, and a subset of adenoviral constructs have been cloned (p53, p53-CC, EGFP control). Major results: Gene therapeutic super p53 (p53-CC) localizes mainly to the nucleus in human ovarian cancer cells (Kuramochi) and exclusively in mouse cells (ID8) as demonstrated by fluorescence microscopy. Preliminary studies indicate that p53-CC causes robust apoptosis in Kuramochi and ID8 cells as well, measured using the 7AAD assay (late stage apoptosis). IC50 values for taxol have been determined in ID8 (and SKOV3) cells. In ID8 cells (which will be used to implant into mice for the syngeneic animal study), p53-CCmut causes the highest levels of apoptosis regardless of whether taxol is added, as seen in vitro.

15. SUBJECT TERMS

Ovarian cancer, gene therapy, p53, modified p53, tumor suppressor, high grade serous carcinoma, combination therapy, carboplatin, paclitaxel, polymeric drug delivery, polymer-adenovirus hybrid

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1. **INTRODUCTION:**

Subject: This project uses a re-engineered p53 (called super p53) for gene therapy that is not subject to dominant negative inhibition by mutated p53 in cancer cells. This super p53 is modified to contain a segment called a coiled-coil domain that allows binding with itself only (resulting in auto-activation). Super p53 cannot bind to other proteins in cancer cells, including wt p53, and subsequently is not inactivated. Purpose: use super p53 for gene therapy of ovarian cancer to allow tumor suppressor/anti-cancer activity. Scope: this super p53 gene therapy can be used in ovarian cancer patients regardless of their p53 status or other genetic heterogeneity. In the proposal and in this report, super p53 = p53-CC (first generation construct) or p53-CCmut (2nd generation construct; also referred to as p53-CC* in some graphs).

2. **KEYWORDS:**

Ovarian cancer, gene therapy, p53, modified p53, tumor suppressor, high grade serous carcinoma, combination therapy, carboplatin, paclitaxel, polymeric drug delivery, polymer-adenovirus delivery

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

Specific Aim 1: Determine if a re-engineered, super p53 (p53-CCmut) is capable of tumor suppressor activity (measured by apoptosis, proliferation, transformative ability and gene expression profiling) and can bypass the dominant negative effect in ovarian cancer cell lines (with varying p53 status) alone, and in combination with standard of care, carboplatin and paclitaxel (CPTX).	**Timeline**	**Percent Completion**	**Site 1**	**Site 2**
Major Task 1: Test activity of p53-CCmut with or without CPTX in ovarian cancer cell lines	Months			
Subtask 1: Test apoptotic activity of p53-CCmut with or without CPTX (caspase 3/7, Annexin V, DNA segmentation, 7-AAD, TUNEL) in cells with varying p53 status. Cell lines used: Kuramochi Caov-4, OVCAR-3, OVCAR4, SKOV3, SKOV3ip, normal cells: BJ, IHOEC	1-8	20% completed; studies ongoing	Dr. Lim	
Subtask 2: Test ability of p53-CCmut with or without CPTX to inhibit cell proliferation and oncogenic potential (trypan blue, MTT, CFA) in cells with varying p53 status. (Same cell lines as subtask 1)	8-10	5% completed; studies ongoing	Dr. Lim	

Subtask 3: Measure p53 gene expression profile of p53-CCmut with or without CPTX Cell line used: SKOV3	10-12	0%	Dr. Lim	
Milestone(s) Achieved: p53-CCmut is capable of killing all ovarian cancer cell lines tested; does not kill normal cells; p53-CCmut activates same genes as wt p53	12		Dr. Lim	Dr. Janát-Amsbury
Local IACUC Approval	3	100%	Dr. Lim	Dr. Janát-Amsbury
Submission of institution approval of animal protocol and related materials for DOD's ACURO approval	3	100%	Dr. Lim	Dr. Janát-Amsbury
Receive ACURO approval before initiating animal experiments	complete	100%	(DOD)	
Specific Aim 2: Deliver super p53 DNA with advanced polymeric systems alone and in combination with CPTX first *in vitro*, then *in vivo* by intraperitoneal injection into a syngeneic orthotopic metastatic mouse ovarian cancer model [33]. 2 polymeric delivery systems will be tested: a. Water soluble lipopolymer (PEG-PEI-cholesterol) [45] currently being used in clinical trials [24, 46] b. RGD-conjugated bioreducible polymer-coated adenovirus (CD-PEG-RGD) The delivery method that provides the highest expression of the gene and highest cell-killing activity *in vitro* will proceed to the *in vivo* testing phase.				
Major Task 2: in vitro testing with polymers				
Subtask 1: deliver p53-CCmut plasmid with PEG-PEI-cholesterol polymer to ID8 ovarian cancer cells; measure transfection efficiency and potency Cell line used: ID8.	13-16	10% completion-WSLP has been synthesized, and ID8 cells tested-Remainder to be completed in year 2	Dr. Lim	Dr. Janát-Amsbury

Subtask 2: construct adenoviral vector containing p53-CCmut; deliver p53-CCmut in adenovirus with CD-PEG-RGD) polymer to ID8 ovarian cancer cells; measure transfection efficiency and potency	13-19	5% completion-some adenoviral constructs have been cloned-Remainder to be completed in year 2	Dr. Lim	Dr. Janát-Amsbury
Milestone(s) Achieved: Determination of optimal polymeric system for in vivo studies	19	To be completed in year 2	Dr. Lim	Dr. Janát-Amsbury
Major Task 3: in vivo testing with optimal polymer				
Subtask 1: establishing primary tumor within ovary	19-21	To be completed in year 2		Dr. Janát-Amsbury (N=63 or 81 animals depending on which polymer is used)
Subtask 2: testing of p53-CCmut using optimal polymeric system with and without CPTX	21-24	To be completed in year 2	Dr. Lim	Dr. Janát-Amsbury
Milestone(s) Achieved: tumor reduction and reduced metastasis with p53-CCmut (with or without CPTX; completion of 1-2 manuscripts (in vitro and in vivo)	24	To be completed in year 2	Dr. Lim	Dr. Janát-Amsbury

- **What was accomplished under these goals?**

Aim 1, Subtask 1: Test apoptotic activity of p53-CCmut with or without CPTX (caspase 3/7, Annexin V, DNA segmentation, 7-AAD, TUNEL) in cells with varying p53 status.

Major activities, specific objectives, results, and tasks yet to do:

Cell Culture: major activities and specific objectives are to obtain, culture, and propagate cell lines described in this proposal.

The cell lines successfully cultured so far include ovarian cancer cell lines:

-Kuramochi (high grade serous carcinoma-HGSC dominant negative p53 with D281Y mutation)

-OVCAR-3 (HGSC, dominant negative p53 with R248Q gain-of-function mutation)

-SKOV3 (derived from ascites, p53 null)

-SKOV3ip1 (more aggressive version of SKOV3, p53 null)- same culture and transfection conditions as SKOV3.

For culture and transfection conditions, see forthcoming data.
Cell lines yet to be grown or in optimization stages:
-Caov-4 (HGSC derived from metastatic site, V147D p53 mutant) ovarian cancer cells require growth without CO_2 and specialized media (still being optimized).
-Normal cells (BJ and IHOEC) also yet to be grown. In Table 1 below (taken from the original proposal), yellow highlight = cells successfully grown and propagated in culture so far.

Table 1. Ovarian cancer cells:	**p53 status**	**BRCA1/2 status**	**Characteristics (all cell lines are human except ID8)** (note: all cell lines are commercially available)
Kuramochi	Dom neg,D281Y	BRCA1 mut	From ovarian cancer ascites; epithelial-like morphology, HGSC
Caov-4	V147D mut	Wt	Ovarian adenocarcinoma; derived from metastatic site (fallopian tube); likely to be HGSC
OVCAR-3	Dom neg, gain of fct, R248Q	Wt	Ovarian adenocarcinoma; epithelial; HGSC
OVCAR-4	L130V mut	Wt	Serous ovarian adenocarcinoma; HGSC; *resistant to platinum*
SKOV3	p53 null	Wt	Ovarian adenocarcinoma; from ascites; not likely to be HGSC
SKOV3.ip1	p53 null	Wt	Ovarian adenocarcinoma; more aggressive version of SKOV3
ID8 cells	p53 null	Wt	Murine ovarian surface epithelial cells spontaneously transformed
Normal cells: BJ, IHOEC	Wt p53	n/a	BJ: Normal fibroblasts; IHOEC: SV40 immortalized ovarian epithelial cells

Subcloning and expression of plasmids: major activities and specific objectives are to successfully subclone, express in *e. coli*, and purify (by maxiprep) plasmids. All plasmids used for this proposal have been made and propagated including:

-p53-CC
-p53-CCmut (p53-CCmutE34K-R55E)
-pEGFP (negative control)
-pEGFP-CC (negative control)
-wt p53 plasmid (positive control in some cases; used for comparison to p53-CC and p53-CCmut constructs.

Transfection optimization of cell lines and initial testing by fluorescence microscopy (to visualize transfected and expressed constructs, and to check for proper subcellular localization): major activities and specific objectives are to optimize transfection conditions and evaluate time points (24-72h) where expression of constructs can be visualized and verified via fluorescence microscopy. Kuramochi (80,000 cells per 4 well chamber) or ID8 cells (40,000 cells per 4 well chamber) were seeded, and 24h later, transfected with 1.25ug plasmid DNA and 1.25ul Lipofectamine 2000. Kuramochi cells were imaged 24h post-transfection, while ID8 cells wereimaged 16h post-transfection. For Hoescht staining, 2ul of 1mg/ml H33342 dye was added

to 500ul media/cells, and rinsed with PBS 15min prior to fluorescence microscopy. Results are shown in Figures 1 and 2, microscopy images of various constructs (listed on left side of diagram). In Kuramochi cells (Figure 1), p53 and p53-CC constructs localize mostly in the nucleus (although p53-CC has some cytoplasmic localization). In ID8 cells (Figure 2), p53 and p53-CC localize to the nucleus as expected. In both cell lines, EGFP and EGFP-CC negative controls localize throughout the cell. In these experiments, we verify that we can transfect 2 cell lines with our plasmid constructs, the proteins are expressed, and localize in the nucleus as expected.

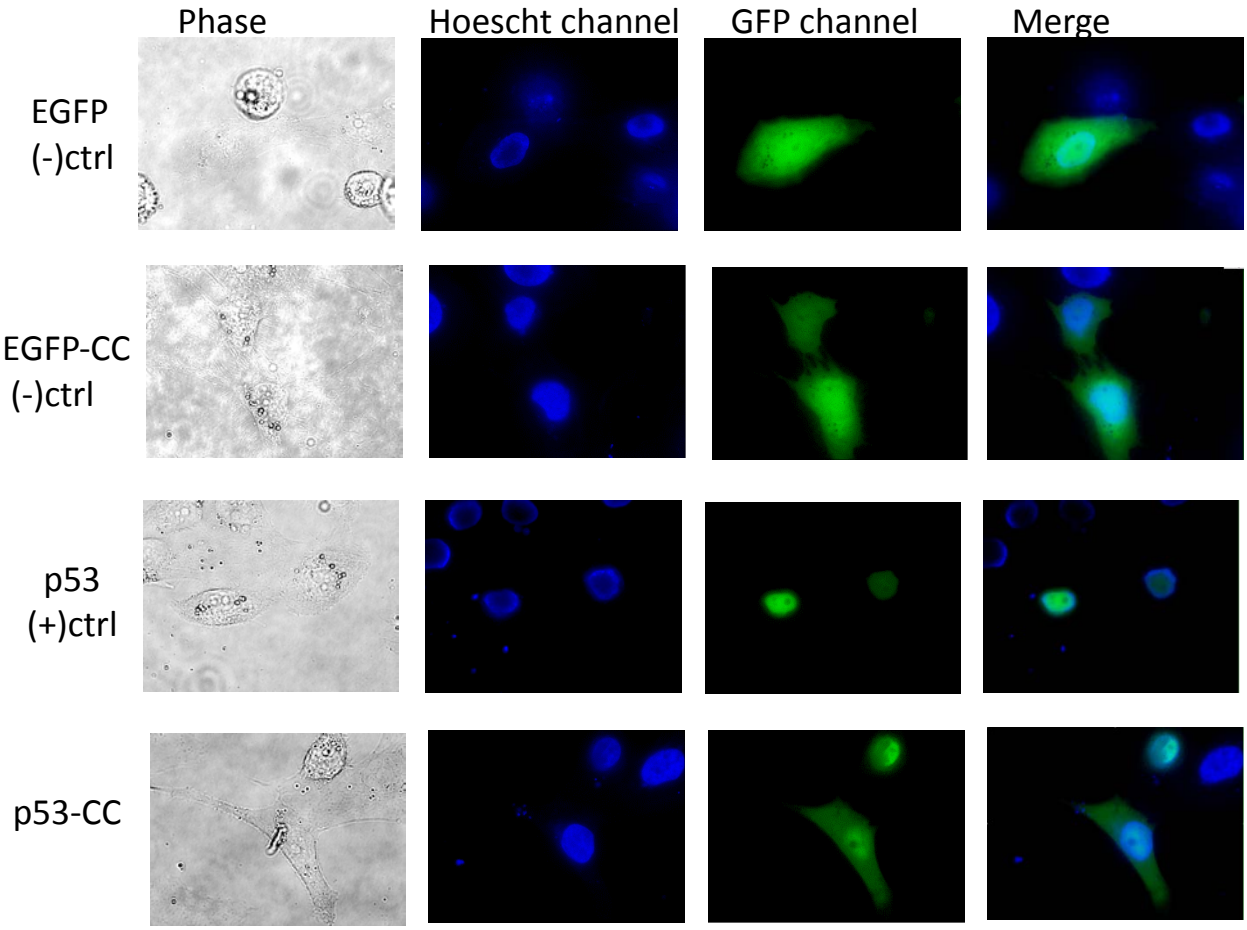


Figure 1. Kuramochi ovarian cancer cells transfected with various constructs (listed on left side). Phase, Hoescht (nuclear staining) channel, GFP channel (all constructs tagged with GFP), and merge panels shown.

Transfection optimization of cell lines and initial testing by measurement of apoptosis (7AAD): major activities and specific objectives are to optimize transfection conditions and evaluate time points (24-72h) where maximal apoptosis of constructs occurs in cell lines measured by 7AAD. Transfections for the 7AAD assay using negative control (EGFP) and positive control (wt p53) have been optimized in Kuramochi, ID8, and OVCAR3 cells. Details are as follows:
Kuramochi cells:
Media: RPMI with 10% heat-inactivated FBS, 1% penicillin/streptomycin/glutamine.
Transfection: 500,000 cells seeded in 6-well plate (for 7AAD), and transfected 24h after seeding

with 5ug plasmid DNA with 5ul Lipofectamine 2000. Cells were stained with 7AAD 48h after transfection, then analyzed and gated for EGFP using the FACSCanto-II/FACSDiva software.

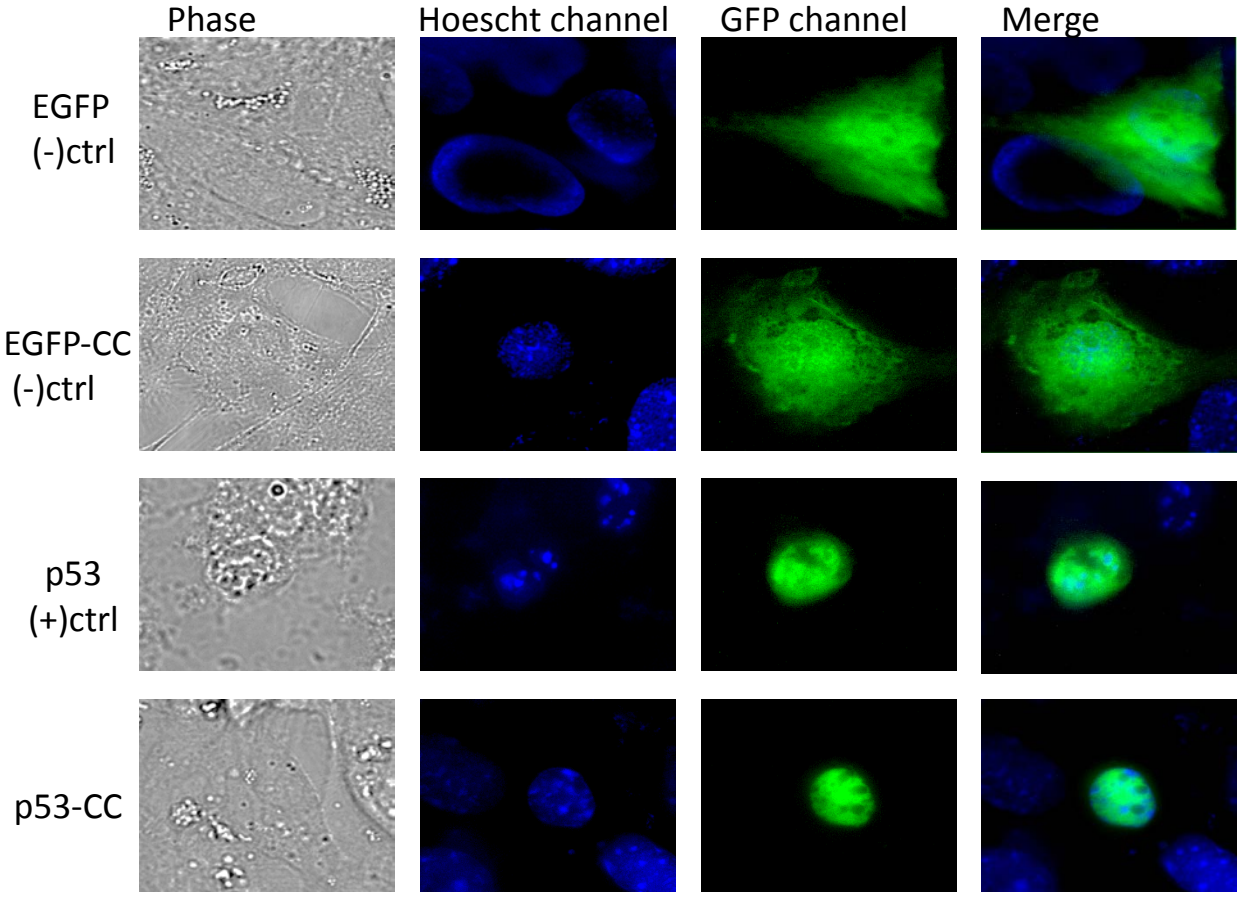


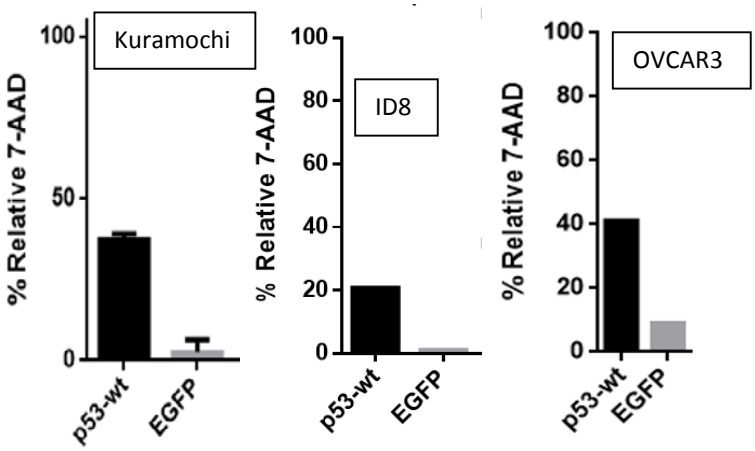
Figure 2. ID8 ovarian cancer cells transfected with various constructs (listed on left side). Phase, Hoescht (nuclear staining) channel, GFP channel (all constructs tagged with GFP), and merge panels shown.

ID8 cells:
Media: DMEM with 5% HI-FBS, 1% penicillin/streptomycin/glutamine, 1% ITSX
Transfection: 125,000 cells seeded in 6-well plate and transfected 24h after seeding with 7.5ug plasmid DNA with 7.5ul Lipofectamine 2000. Cells were stained and analyzed as above.

OVCAR3 cells:
Media: RPMI with 20% FBS, 1% penicillin/streptomycin/glutamine, 1% bovine insulin.
Transfection: 250,000 cells seeded in 6 well plate and transfected 24h after seeding with 5ug plasmid DNA and 5ul Lipofectamine 2000. Cells were stained and analyzed as above.

Figure 3 shows preliminary data for these 3 cell lines; in all cases, wt p53 shows apoptotic activity, while EGFP control has low activity, as expected. The apoptotic activity of these constructs still needs to be tested in the other cell lines mentioned in the proposal.

Figure 3. 7AAD activity plots for Kuramochi, ID8, and OVCAR 3, respectively. Positive control (p53 wild type) and negative control (EGFP) tested so far (n = 3 when error bars shown, otherwise n = 2).



We have also just started experiments in Kuramochi cells with the p53-CC construct as in Figures 4A and importantly, also in ID8 cells in Figure 4B. In Figure 4B, the p53-CCmut (p53-CC*) demonstrates activity in vitro, a critical piece of data that bodes well for *in vivo* studies.

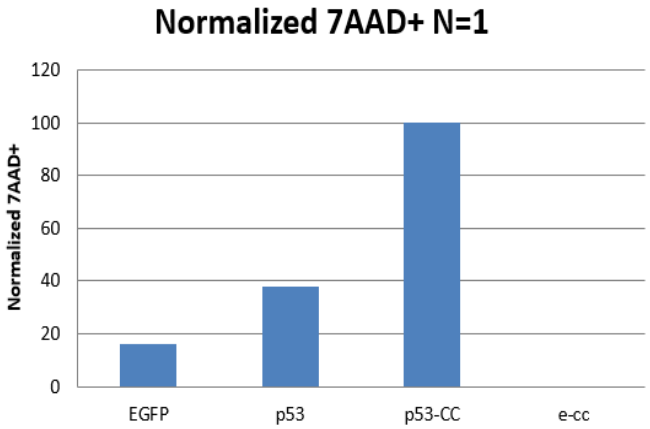


Figure 4A. Preliminary apoptotic activity of various constructs in Kuramochi cells. p53-CC construct appears to have robust apoptotic activity compared to other constructs. (e-cc = EGFP-CC)

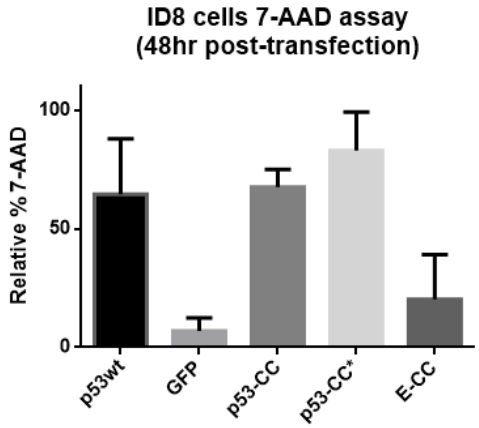
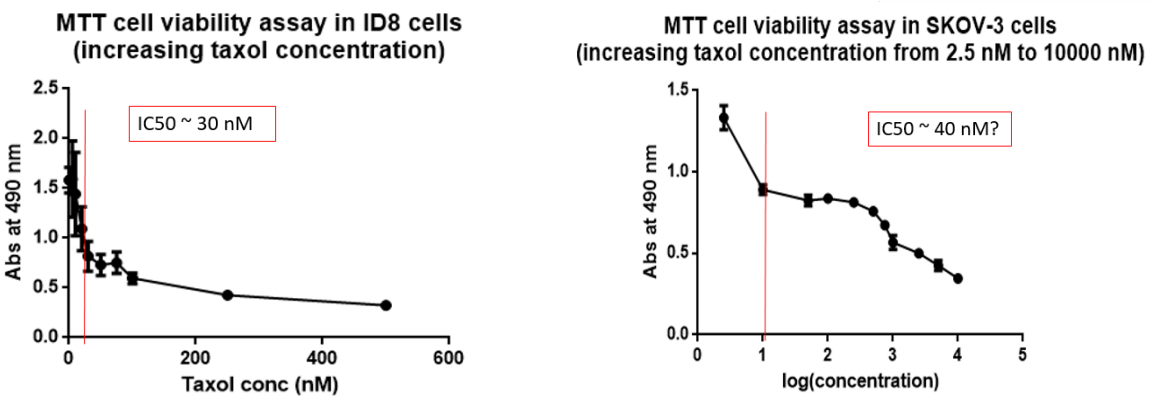


Figure 4B. Apoptotic activity of various constructs in ID8 cells. p53-CC* (final p53-CCmut construct) appears to have robust apoptotic activity compared to other constructs. (E-CC = EGFP-CC negative control)

Aim 1, Subtask 2: Test ability of p53-CCmut with or without CPTX to inhibit cell proliferation and oncogenic potential (trypan blue, MTT, CFA) in cells with varying p53 status.
Major activities, specific objectives, results, and tasks yet to do:

Determining estimates of IC50 values for paclitaxel and carboplatin in ovarian cancer cell lines: major activities and specific objectives are to determine 50% inhibitory concentrations of paclitaxel (taxol) and carboplatin in ovarian cancer cell lines. This was performed by adding increasing drug doses to cells, and measuring cell death using the MTT cell viability assay. So far, IC50's for ID8 cells ~30nm, and for SKOV-3 cells, ~40nM. IC50's for other cell lines yet to be tested, and carboplatin IC50's will be tested in all cell lines. Carboplatin initially was problematic since the first time we purchased it (Sigma), the drug was inactive (ie, killing was not occurring at even very high doses). We will repurchase from another vendor and will re-test.

Figure 5. Determination of approximate IC50 in ID8 and SKOV-3 cells.



Combination studies with our gene constructs and taxol, with carboplatin, and with both drugs (CPTX): major activities and specific objectives are to determine if combination gene and drug treatment result in increased apoptosis of ovarian cancer cells *in vitro*. We have started transfecting cells with our gene constructs, p53-CC and/or p53-CCmut (also known as p53-CC*). Gene constructs are first transfected into cells, then drug (taxol to start) is added, and the 7AAD apoptosis assay is performed 48h later. Kuramochi, ID8, and SKOV3 were the first cell lines tested as shown in Figures 6-8. For Figures 6 and 8, EGFP gated (green bars) and all cells (red bars) shown in graph; for Figure 7, only EGFP gated cells shown. The pilot data in these

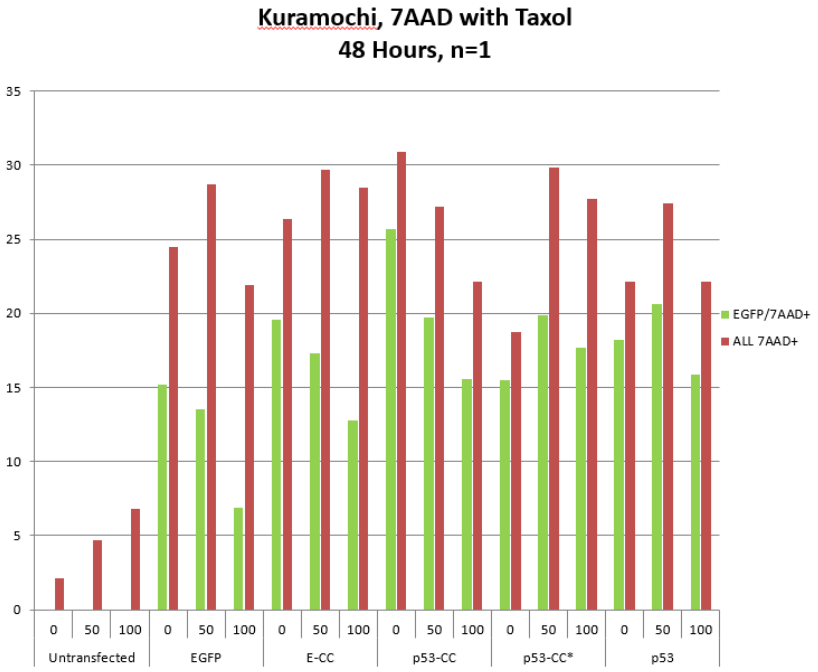


Figure 6. Kuramochi cells with various treatments. Untransfected = no gene transfected. EGFP, EGFP-CC, p53-CC, p53-CCmut (*), and p53 transfected cells were treated with 0, 50, or 100nM doses of taxol.

figures is fairly complex, and we note differences in effect between all 3 cell lines. These experiments must be repeated in triplicates before complete analyses can be made; however some trends can be gleaned from these pilot experiments. Note are that in Kuramochi cells, taxol displays a typical dose related effect, and transfection may be contributing to cell death (regardless of what plasmid is transfected, Figure 6). In ID8 cells, the p53-CC (or p53) alone causes robust apoptosis, irrespective or taxol added (Figure 7). In Figure 8, it appears that taxol may "interfere" with transfections (and hence the ability of these constructs to cause apoptosis), so the timing of dosing must be optimized.

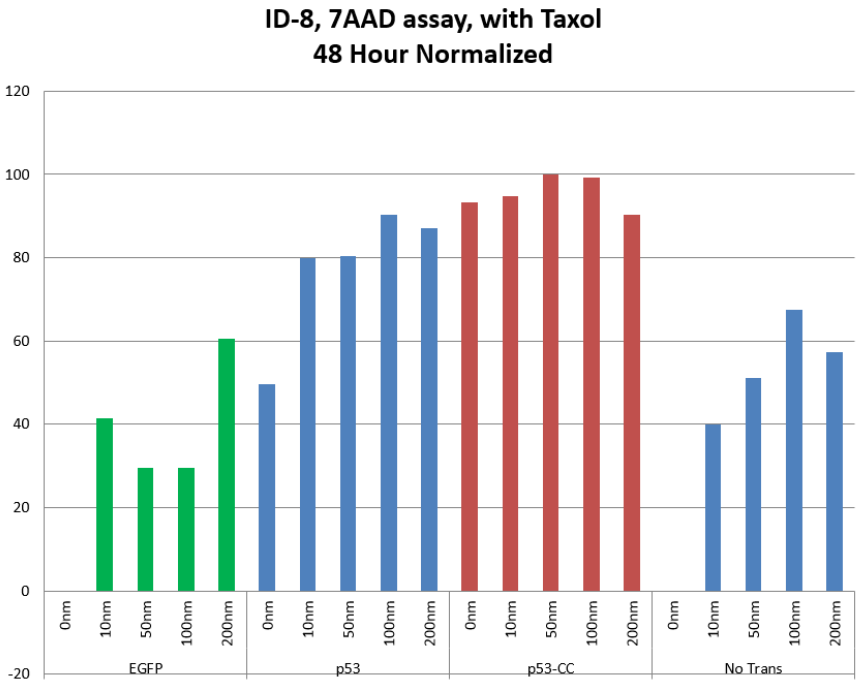
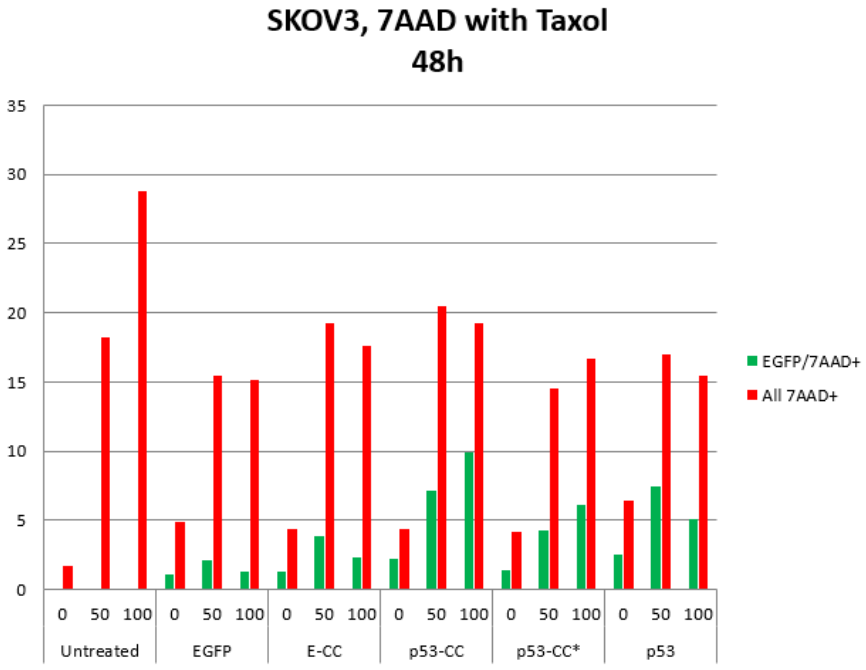


Figure 7. ID8 cells with various treatments. Untransfected = no gene transfected. EGFP, p53, or p53-CC transfected cells or untransfected cells were treated with 0, 50, or 100nM doses of taxol.



SKOV3 cells were grown in DMEM with 10% HI FBS, 1% penicillin/streptomycin/L-glutamine. For 7AAD assay, used 250,000 cells plated per 6 well plate, transfected 24h later using 5ug plasmid DNA and 5ul Lipofectamine 2000.

Figure 8. SKOV3 cells with various treatments. Untreated = no gene transfected, EGFP, EGFP-CC, p53-CC, p53-CCmut (*), or p53 transfected cells were treated with 0, 50, or 100nM doses of taxol.

Lastly, WSLP polymer has been successfully synthesized by Dr. Nam (post doc in S.W. Kim's lab), and some of the plasmid constructs have been cloned into adenovirus (p53, p53-CC, EGFP control) in preparation for *in vivo* work.

Summary:
In this report, we show preliminary data indicating that gene therapy using re-engineered super p53 (p53-CC constructs) is effective in killing ovarian cancer cells *in vitro*. This is unreported, novel finding paves the way for using super p53 for ovarian cancer treatment. **Main activities and objectives** completed to date include optimization of cell culture growth and determining transfection conditions for human ovarian cancer cell lines, including Kuramochi, OVCAR3, SKOV3, SKOV3.ip1, and mouse ovarian cancer cells (ID8). 7AAD and MTT assay conditions have been optimized. WSLP (polymer) has been successfully synthesized, and a subset of adenoviral constructs have been cloned (p53, p53-CC, EGFP control). **Major results**: Gene therapeutic super p53 (p53-CC) localizes mainly to the nucleus in human ovarian cancer cells (Kuramochi) and exclusively in mouse cells (ID8) as demonstrated by fluorescence microscopy. Preliminary studies indicate that p53-CC causes robust apoptosis in Kuramochi and ID8 cells as well, measured using the 7AAD assay (late stage apoptosis). IC50 values for taxol have been determined in ID8 (and SKOV3) cells. In ID8 cells (which will be used to implant into mice for the syngeneic animal study), p53-CCmut causes the highest levels of apoptosis regardless of whether taxol is added, as seen *in vitro*.

- **What opportunities for training and professional development has the project provided?**
- Nothing to report (Informally, grad student has presented data from this project in lab meeting multiple times, and the project has been discussed in terms of troubleshooting and direction. New lab members are being trained on the project, and students will present their work in departmental seminar and at a national meeting in the near future).
- **How were the results disseminated to communities of interest?**
  - Nothing to report yet, but grad student will present work in departmental seminar and a national meeting.
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Now that the basic groundwork for the experimental assays has been laid (cell culture, transfections, apoptosis assays, combination studies), the team (see below under "actual or anticipated problems or delays and actions or plans to resolve them") including Ben Bruno (senior grad student), Erica Van Der Mause (new PhD student), Julian Knerr (German exchange student, and Joe Cho (MD/PhD student) will be assigned tasks (ie, 1 assay, several different cell lines) to systematically and methodically complete the *in vitro* work. Students will report experimental results to PIs (Lim and Amsbury) as soon as they have been completed, and troubleshooting will occur as they go. The first priority of *in vitro* experiments will on the ID8 cells, since they will inform the *in vivo* work in aims 2-3. Once the in vitro work has been completed, the adenoviral versions of this will be tested *in vivo* by our animal technician Youngen Sun, who has years of experience with the syngeneic mouse model. Cell work will continue simultaneously with the animal work if not complete by then.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
- Nothing to report yet, but when published/completed may provide a new gene therapeutic/combination therapy for ovarian cancer.
- **What was the impact on other disciplines?**
- Nothing to report.
- **What was the impact on technology transfer?**
- Nothing to report.
- **What was the impact on society beyond science and technology?**
- Nothing to report yet.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

  No changes in approach/objectives/scope.
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - We were unable to hire a full time student at the onset of the project. After an unsuccessful attempt to hire a post-doc, we shifted one graduate student to this project (Ben Bruno), and have hired a new full time graduate student (Erica Van Der Mause, started May 2016) who will complete the bulk of the project. We also have a full time German exchange student (Julian Knerr) who started in April who is currently being trained on this project (pharmacy rotation, unpaid 6 month position), and a MD/PhD student (Joe Cho, who has completed his PhD at the Huntsman Cancer Institute) who will also assist on this work on his 1 semester unpaid research rotation in my laboratory starting in August. Now that we have completed pilot experiments and have a good grasp of the technical aspects of the project, we will move forward towards completion. For the animal studies, we will utilize the expertise of Dr. Janat Amsbury's animal technician, Youngen Sun (as planned originally). Due to the delays so far, we will prioritize the experiments using ID8 cells so we can advance to the animal studies in a timely fashion. Along with animal studies, our team will continue to work on the *in vitro* studies in the other cell lines in this proposal.
- **Changes that had a significant impact on expenditures**
  - Delays in hiring staff had a significant impact on expenditures; we were not able to hire a qualified graduate student, so instead I moved one graduate student onto this project part time, and hired another student to work on this full time (started May 2016). We will be therefore back on track with expenditures.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  - Nothing to report.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**

  Nothing to report.
  - **Journal publications.**
  - Nothing to report.

- **Books or other non-periodical, one-time publications.**
- Nothing to report.
- **Other publications, conference papers, and presentations.**
- Nothing to report.

- **Website(s) or other Internet site(s)**
  Nothing to report.
- **Technologies or techniques**
  Nothing to report.
- **Inventions, patent applications, and/or licenses**
  Nothing to report.
- **Other Products**
  Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	*Carol Lim*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*1.5*
Contribution to Project:	*Lim has directed the entire project thus far, and has been involved with experimental design, troubleshooting, and analysis of data.*
Funding Support:	*National Institutes of Health, NCI*

Name:	*Margit Janat Amsbury*
Project Role:	*Key Collaborator, Co-I*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*1.5*
Contribution to Project:	*Dr. Amsbury has provided assistance with cell lines, drugs, and planning experiments.*
Funding Support:	*National Institutes of Health, NCI*

Name:	*Benjamin Bruno*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*6*
Contribution to Project:	*Bruno has designed, conducted, troubleshot, and analyzed the in vitro experiments in this proposal.*
Funding Support:	*n/a*

Name:	*Erica Van Der Mause*
Project Role:	*Graduate Student, starting May 17*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*12*
Contribution to Project:	*Will design, conduct troubleshoot, and analyze the in vitro experiments in this proposal (training by Bruno)*
Funding Support:	*n/a*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

- A new grant is now active, NIH R21 CA187289-02 (07/07/2015 - 06/30/2017) with no changes in level of effort for this DOD grant. Lim is PI and Janat-Amsbury is Co-I.

- **What other organizations were involved as partners?**
  - Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:** *not applicable*
   - **QUAD CHARTS:** *not applicable*

9. **APPENDICES:** *not applicable*

********** **ADDITIONAL NOTES:** not applicable